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TITLE: Differential Expression of DNA Double-Strand Break Repair  
Proteins in Breast Cells

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<b>13. ABSTRACT (Maximum 200 Words)</b>  Two mechanisms that repair DNA double-strand breaks in mammalian cells are homologous recombination and non-homologous DNA end-joining (NHEJ). Previous studies showed that a critical component of the NHEJ pathway, the DNA-activated protein kinase (DNA-PK), was poorly expressed in non-lactating (resting) breast tissue. Therefore, we proposed to identify the mechanisms responsible for regulating levels of non-homologous end-joining DNA repair components in human breast tissue and to measure the DNA double-strand break repair capacity of breast epithelial cells.  We reexamined the expression of DNA-PK in human breast tissues by immuno-histochemistry and extended these studies to two other components of the NHEJ repair pathway, XRCC4 and DNA ligase IV, as well as three other DNA repair components NBS1, MRE11, and PCNA. In contrast to the original report, 90% of the epithelial cells in normal resting breast tissues from 10 different patients express both components of DNA-PK, DNAPKcs and Ku. These tissues also expressed XRCC4, DNA Ligase IV, NBS1, MRE11, and PCNA at similar levels. However, only PCNA was expressed in the stromal cells from the same tissues. Somewhat strong expression of DNA-PK was seen in epithelial cells. Surprisingly, however, only PCNA was detectable expressed in stromal cells from these same tissues.				
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## Introduction

DNA double-strand break (DSB) repair is critical for cell survival and for preventing genome rearrangements leading to cancer. In mammalian cells, it is carried out by two mechanisms, homologous recombination and non-homologous DNA end joining (NHEJ), but our knowledge of both the processes *in vitro* and *in vivo* is limited. We recently observed that DNA-activated protein kinase (DNA-PK), a critical component of NHEJ is poorly expressed in non-lactating (resting) breast tissue compared to most other tissues especially in lactating breast tissue where it is expressed at high levels (Moll et al, 1999). All tissues possess similar amounts of the mRNAs for the three polypeptide components that comprise DNA-PK (DNA-PKcs, Ku70 and Ku80), suggesting that the poor expression of DNA-PK in resting breast epithelial cells results from posttranscriptional mechanisms rather than transcriptional regulation. Our findings suggested that resting breast tissue might be compromised for the repair of DSBs compared to other tissues. Therefore we proposed to identify the mechanisms responsible for regulating levels of NHEJ DNA repair components in human breast tissue and measure the DNA double strand break repair capacity of breast cells. To accomplish these goals, we proposed to- I) Establish culture conditions that recapitulate DNA-PK and NHEJ component expression in breast tissue. II) Examine the effects of low and high expression levels on DNA repair and genome stability. III) Identify the mechanisms that regulate NHEJ component expression in breast tissue.

## Body

The Objectives of this project were based on our findings in Moll et al. (1999) where mammary epithelial cells of the lactating human breast tissue expressed high levels of both DNA-PK and Ku proteins while none of these proteins were detected in epithelial cells of the normal (resting) human breast tissue.

These findings were recapitulated by analyzing the paraffin embedded breast tissue sections by immunohistochemistry using antibodies specific for the two subunits of DNA-PK, DNA-PKcs, the catalytic subunit, and Ku, the targeting/regulatory subunit. The studies were extended further to the other double-strand break (DSB) repair proteins of non-homologous DNA-end joining (NHEJ) pathway, XRCC4 and DNA ligase IV. The findings are summarized in Table 1. The micrographs of selected breast tissue sections stained with antibodies to various DNA repair proteins are shown in Appendix 1.

Both resting as well as lactating breast tissue sections of normal women showed strong expression of DNA-PKcs, Ku70/80, XRCC-4, DNA Ligase IV as well as NBS1 and MRE11. DNA-PK showed strong nuclear staining while Ku was strongly cytoplasmic. The rest of the proteins showed nuclear expression similar to DNA-PKcs. However as is shown previously, a striking cell-to-cell specificity was observed. The expression of all the proteins was restricted to epithelial cells including both the myoepithelial and luminal epithelial cells. The stromal cells in all the cases showed no or very little if any expression of these proteins. Within epithelial cells variability in the intensity of DNA-PK of expression was observed. In 3 out of 8 cases, about 30% of epithelial cells were negative whereas the rest were strongly positive.

**Table 1: Expression of NHEJ double strand break repair proteins in Breast Tissue samples:**

Proteins	Resting Breast Tissue		Lactating Breast Tissue	
	Epithelial cells	Stromal cells	Epithelial cells	Stromal cells
DNA-PK (Ab-1)	++ (90%)	+/-	+++	-
DNA-PK (Ab-2)	++ (90%)	-	+++	-
Ku 70/80	++ (90%)	-	++	-
XRCC4	++ (80%)	-	++	-
DNA Ligase IV	++ (80%)	-	++	-
NBS1	++ (80%)	-	++	-
MRE11	++ (80%)	-	++	-
PCNA	++ (100%)	++	++	++
Mib (Ki67)	-	-	-	-

®Visual estimate of nuclear staining intensity (+/- to +++). The percentage of positive cells is estimated where staining and cell type distribution is uniform. 10 fields/slide were screened and average positivity determined.

The above findings were confirmed using more than one antibodies in case of DNA-PKcs. The two antibodies are directed against different epitopes of the protein. Also over 10 different pathology cases were analyzed each for resting and lactating breast tissue samples. To determine if there is differential expression of these proteins over the wide time span of pregnancy, cases from different developmental stages of pregnancy were selected and examined for DNA-PKcs, KU80 and XRCC4. All these cases of resting as well as lactating breast tissue sections were also examined for DNA Ligase IV, MRE11 and NBS1. MIB (ki-67) was used as the negative control marker and PCNA was used as the positive control marker throughout this analysis; they showed minimal and maximal positivity, respectively.

The interesting observation emerging from our analysis is that the stromal cells are devoid of, or have very low levels, of any of these proteins. This intriguing phenomenon is currently being examined in more details. A representative stromal cell line Hs 574.T was obtained from ATCC (cat.# CRL-7345). This cell line is of fibroblast type derived from the ductal carcinoma of human mammary gland and appeared to be of stromal origin.

Expression of DNA-PK as well as other NHEJ repair proteins is being studied in this cell line by Immunofluorescence (IF) and Western immunoblot analysis using the available antibodies specific for each of the NHEJ proteins. Our preliminary studies suggest that very low levels, if any, of DNA-PKcs is expressed in this cell line. The activities of DNA-PK and XRCC4/Lig IV will be assayed in these cells. Genomic instability studies also will be carried out.

Recently, Okayasu et al. (2000) reported that DNA-PKcs and Ku were expressed at lower levels in mouse breast tissues than in other mouse tissues. They further showed that BALB/c mice, which are 20 times more susceptible to low-level radiation induced breast cancer than most other mouse strains (e.g. C57Black) expressed less DNA-PKcs protein than resistant mouse strains. However, Ku expression was similar in both sensitive and resistant strains. Further, breast cancer susceptibility was associated with DNA double-strand break repair capacity

radiosensitivity, and DNA-PKcs expression. More recently, two differences in the amino acid sequence of DNA-PKcs from BALB/c mice compared to C57Black mice were discovered (Yu et al., 2001). Although DNA-PK has been implicated in the cellular response to ionizing radiation in mammalian cells (Muller et al., 1999), no correlation has yet been seen between radiosensitivity in cancer patients and the expression and activities of DNA repair proteins in the fibroblast cell lines derived from these patients (Carlomagno et al., 2000). Nevertheless, it has been reported that more than cell lines from 40 percent of human breast cancer patients exhibit increased genome instability (Scott et al., 1994).

This could be due to the limitations of the detection techniques used in the study (immunohistochemistry and western blot), and we feel that we might be able to establish the correlation by using more sensitive assays such as Flow cytometric analysis (FACS) or quantitative IF. FACS will be carried out on lymphocytes/lymphocytic cultures established from patients with different radiosensitivities. Furthermore, the mouse studies described above indicate that polymorphisms in NHEJ genes can result in decreased expression levels, which, in turn, may result in increased sensitivity to ionizing radiation and breast cancer susceptibility. We therefore suggest exploring this possibility by examining the expression of NHEJ proteins in cell lines derived from breast cancer patients that do or do not exhibit genome stability.

### **Key Research Accomplishments**

- Reexamined expression of DNA-PKcs and Ku80 in epithelial cells from resting human breast tissue; both were expressed.
- Examined expression of NHEJ components XRCC4 and DNA ligase IV in epithelial cells from resting human breast tissue; both were expressed
- Examined expression of repair components NBS1 and MRE11 in epithelial cells from resting human breast tissue; both were expressed.
- Examined expression of above components in stromal cells from resting and lactating human breast tissue; none were expressed.
- Identified a human breast stromal cell line that recapitulates lack of expression of DNA repair proteins.

### **Reportable Outcomes**

None to date (manuscript in preparation)

### **Conclusions**

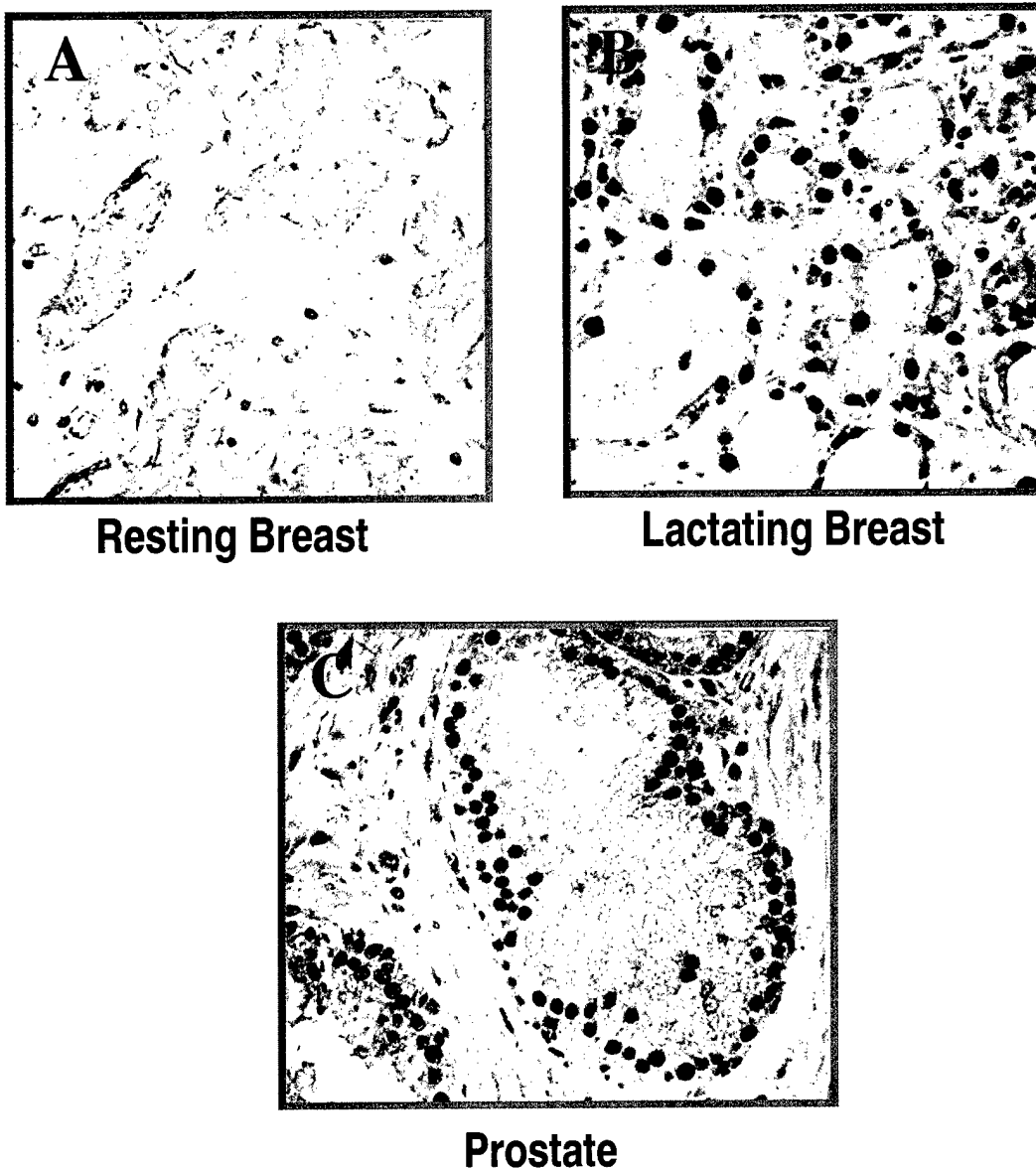
Both resting as well as lactating breast tissue sections of normal women showed strong expression of DNA-PK, Ku70/80, XRCC4, DNA Ligase IV as well as NBS1 and MRE11 in epithelial cells. Because histocytochemistry is at best a semi-quantitative method, we cannot conclude that these components are expressed a equal levels in epithelial cells from resting breast tissue compared to epithelial cells in lactating breast tissue or in other tissues; however, these new observations suggest that differences in the level of these repair proteins will at best be

minimal. Nevertheless, our new observations show that stromal cells from breast tissue showed no or very little if any expression of these proteins.

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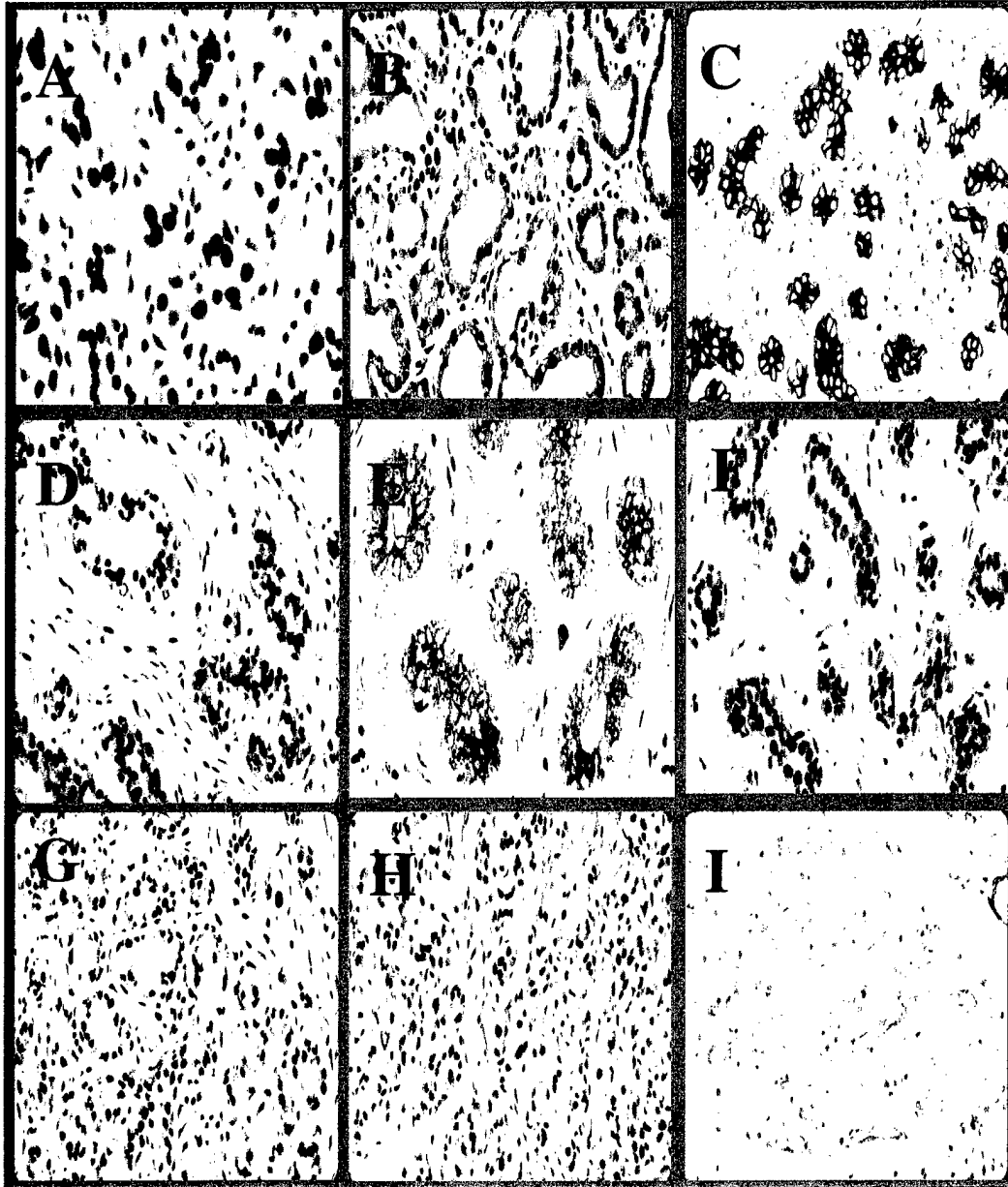
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Original observations from Moll et al. (1999)



**Figure 1. Expression of DNA-PK<sub>cs</sub> in normal human tissues.** (A) resting breast, lobule with acini; (B) lactating breast, milk-filled acini; (C) prostate. Immunohistochemical detection was with DNA-PK<sub>cs</sub> antibody AB-145 (Oncogene Research Products, Cambridge, MA), a rabbit polyclonal serum that recognizes epitopes between amino acids 2015 and 2134 of the 4127 residue polypeptide. Original magnification was 40X. From Moll et al., (1999).





**Figure 2. Expression of DNA PK and other NHEJ DSB repair proteins in human breast tissue.** A. DNA-PKcs in resting breast tissue; B. DNA-PKcs in lactating breast tissue; C. Ku80; D. XRCC4; E. DNA Ligase IV; F. Mre11; G. NBS1; H. PCNA; I. Mib(Ki67).

**Methods.** Tissues came from the routine pathology archives of the Department of Pathology, University Hospital at SUNY Stony Brook. After biopsy or resection, the tissues were fixed in 10 % formalin for up to 18 h, and processed for light microscopy by standard methods. Immunohistochemical staining was as described in Moll et al. (1999). Briefly, 4  $\mu$ m paraffin sections were deparaffinized by microwaving sections in 100 mM citric acid buffer, pH 6.0 for 5 min 6 times. Sections then were treated with 0.3 %  $H_2O_2$ /methanol to quench endogenous peroxidase activity. After blocking with 10 % normal goat serum, sections were incubated at 4°C overnight with primary antibody in 2 % bovine serum albumin/phosphate buffered saline. Biotinylated goat anti-mouse or goat anti-rabbit secondary antibodies and streptavidin/biotin complex were applied for 30 min each (ZYMED, San Francisco CA), followed by 8 min incubation in diaminobenzidine substrate and extensive washing. Sections were lightly counter-stained in hematoxylin and mounted under a coverslip. Sections were photographed with a Nikon photomicroscope.